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Research Article

Novel complexes of cyclin-dependent kinases and a cyclin-like protein from *Arabidopsis thaliana* with a function unrelated to cell division

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Abstract. Although the majority of cyclin-dependent kinases (CDKs) play a key role in cell cycle progression, recent evidence has shown that CDKs are also implicated in transcription regulation. Here, we describe two *Arabidopsis* CDKs designated Arath;CDKC;1 and Arath;CDKC;2. These CDKs share a PITAIRE signature in the cyclin-binding domain and the structural characteristics of mammalian CDK9. Yeast two-hybrid screens and immunoprecipitation assays identified CDKC-interacting proteins with homology to the animal cyclin T/cyclin K group. We suggest that these *Arabidopsis* CDKCs may be part of a kinase complex similar to the animal positive transcription elongation factor b, whose activity is essential for transcription control. Expression studies showed that Arath; *CDKC* transcripts are mainly confined to epidermal tissues and are most abundant in flower tissues. No expression was detected in actively dividing *Arabidopsis* tissues, suggesting a role for the CDKC proteins in differentiated cells.

Key words. *Arabidopsis thaliana*; cell cycle; cyclin-dependent kinase (CDK); cyclin T; transcription control; two-hybrid interaction.

Cyclin-dependent kinases (CDKs) have been identified as major cell cycle regulators and considerable progress has been made on their characterization. CDK activity has been shown to depend on their interaction with regulatory proteins known as cyclins.

In *Arabidopsis thaliana*, two CDKs have been identified that clearly play a role in cell cycle regulation [1]. These CDKs have recently been designated Arath;CDKA;1 and Arath;CDKB1;1 and are representatives of two major plant CDK families, the CDKA and CDKB groups [2]. The CDKA group has a characteristic PSTAIRE motif and is involved in cell proliferation and maintenance of cell division competence in non-proliferating tissues [3]. The CDKB group contains a PPT(A/T)LRE motif that is found only in plant CDKs. The CDKB1;1 protein has been shown to play a specific role in G2/M transition [4].

Although most CDKs in association with their cyclin partners are involved in cell cycle regulation, other CDK/cyclin complexes have been found to govern multiple cellular pathways, including signal transduction and differentiation. Some complexes, such as CDK7/cyclin H, CDK8/cyclin C, and CDK9/cyclin T, are responsible for the control of the transcription machinery in animals [5].

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The elongation control of nascent transcripts plays a major role in the expression regulation of most genes. The conserved carboxyl-terminal domain (CTD) of the RNA polymerase II (RNAP II) large subunit is a docking place for positive and negative elongation factors. A determining role in processive elongation has been attributed to the positive transcription elongation factor b (P-TEFb) complex. P-TEFb has been proposed to determine the fate of RNAP II at the early phase of elongation, causing RNAP II to become competent to produce full-length transcripts. In animal cells, phosphorylation and dephosphorylation of the conserved CTD domain of RNAP II seem to be the key regulatory steps that control the entry of this RNA polymerase into the elongation process [6, 7]. P-TEFb is a CDK complex that consists of CDK9 (also referred to as PITALRE kinase) and one of several cyclins, including T1, T2, and K [8–10].

The elevated levels of CDK9 and its regulatory subunits (cyclins T1 and T2) in terminally differentiated cells, together with the fact that CDK9/cyclin T complexes are not cell cycle regulated, distinguish CDK9 from the majority of other CDKs [11]. Furthermore, because cyclin T levels are not cell cycle regulated, CDK9 kinase activity does not change during the different phases of the cell cycle. Moreover, unlike the other CDKs, which regulate cell cycle progression, CDK9 fails to phosphorylate H1, but it phosphorylates the C terminus of the protein product of the retinoblastoma gene (pRb) [12].

We characterized a new *A. thaliana* CDK group, designated Arath;CDKC. Yeast two-hybrid screening revealed that Arath;CDKC;2 interacts with a protein homologous to the animal cyclin T. The formation of CDKC/cyclin T complexes in *Arabidopsis* was confirmed by the binding in vitro of both CDKC;1 and CDKC;2 to the cyclin T protein during immunoprecipitation**.** Association of the Arath;CDKC proteins with a cyclin T-like protein indicates that plant CDKCs might form a kinase complex with functional characteristics of the animal P-TEFb. Based on their sequence similarities to the animal CDK9 and binding to a cyclin T-like protein, we propose that the *Arabidopsis* CDKC proteins are functional homologues of the animal CDK9.

Materials and methods

cDNA cloning

Arath;*CDKC;2* cDNA was isolated from cell suspension cultures of *A. thaliana* (L.) Heynh., using sequence information of expressed sequence tag (EST) for primer design. Full-length cDNA was cloned by 5′-end amplification, with the 5′-end CapFinder Kit according to the manufacturer's protocol (Clontech, Palo Alto, Calif.). A second *Arabidopsis* gene coding for a putative CDKC protein (Arath;*CDKC;1*) was cloned based on its sequence homology to the identified *CDKC;2*.

Sequence analysis and phylogeny

Alignment was edited and reformated with BioEdit [13]. Similarity between proteins was based on a BLOSUM62 matrix [14]. Phylogenetic analysis was performed on the more conserved positions of the alignment. A phylogenetic tree was constructed with the neighbor-joining algorithm using the software package TREECON [15].

Yeast two-hybrid assays

Library screening was performed as described previously [16]. Vectors and strains (HF7c) were provided by the Matchmaker two-hybrid system (Clontech). The Arath;*CDKC;2* bait was constructed by cloning a polymerase chain reaction (PCR)-amplified *CDKC;2* fragment (740 bp) cut with *Eco*RI/*Bam*HI, in frame with the *GAL4* DNA-binding domain of the pGBT9 vector (Clontech), which was digested with the same enzymes.

For additional pairwise two-hybrid assays using *cyclin T*, the corresponding full length-coding region was inserted into a GATEWAY vector (Invitrogen, Carlsbad, Calif.) containing the *GAL4* activation domain. The *cyclin* gene was inserted by homologous recombination between the *attB* sequence in the GATEWAY vector and at both ends of the *cyclin T* fragment, which was amplified by PCR with primers containing the terminal *attB* sites (according to the manufacturer's instructions).

Plasmids encoding baits and preys were co-transformed into a yeast reporter strain and their association was determined by the ability of the co-transformed strains to grow on histidine-deficient medium.

In vitro transcription/translation and immunoprecipitation

Influenza hemagglutinin (HA)-tagged CDKC;1 and CDKC;2 were constructed by cloning into the pSK plasmid (Stratagene, La Jolla, Calif.) containing the HA-tag [17]. The coding sequences of both genes were amplified by PCR and cloned into the *Eco*RI and *Bam*HI sites of the pBluescript plasmid (Stratagene) containing an HA-tag (HA-pSK). The *c-myc*-tagged *cyclin T* and *DAG-like* (DAG; differentiation and greening) genes were generated by PCR amplification of the respective coding sequences, followed by cloning into the *Eco*RI and *Spe*I sites of pSK containing a doubled *c-myc* tag (*c-myc*-pSK). All cloning steps were carried out according to standard procedures. The reading frames were verified by direct sequencing.

The mutant forms of HA-tagged *CDKC;1* and *CDKC;2* ($CDKC^{1-217}$) as well as the deleted versions of *c-myc*-tagged *cyclin T* ($cycT^{1-153}$ and $cycT^{1-213}$) were obtained by PCR amplification of the respective full-length versions cloned in pSK (see above), using T7 (forward) primer and specific end (reverse) primers.

In vitro transcription/translation was performed using the TNT T7-coupled wheat germ extract kit (Promega, Madi-

son, Wisc.) primed with appropriate plasmids for 2 h at 30 °C. For immunoprecipitation, an aliquot of each TNT sample (10 µl of 50 µl) was mixed and diluted at 1 : 5 in Nonidet P40 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin/aprotinin/pepstatin) and incubated for 2 h at 4 °C with anti-c-myc (9E10; BabCo-Covance, Berkeley, Calif.) or anti-HA (HA.11; Babco-Covance) antibodies. Protein-A-Sepharose beads [40 µl 25% (v/v)] were added and incubated for 1 h at 4 °C. The beads were then washed four times with Nonidet P40 buffer. Immune complexes were eluted with 10 µl 2 × sodium dodecyl sulfate (SDS) sample buffer and analyzed by 12% or 15% SDS-polyacrylamide gel eletrophoresis (PAGE) and by autoradiography. These experiments did not attempt to precisely map the region of interaction between the CDK and cyclin partners under study, but to better characterize and consolidate the existence of a truly new CDK/cyclin complex.

Reverse transcriptase-PCR analysis

Total RNA was extracted from young seedlings, roots, rosettes, stems, and flowers of *A. thaliana* ecotype Columbia according to standard protocols. Two micrograms of each sample was reverse-transcribed (RT) into cDNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Gaithersburg, Md.). Semi-quantitative RT-PCR amplification of the cDNA was carried out in a LightCycler real-time PCR (Roche Diagnostics, Brussels, Belgium) with the gene-specific primers 5′-ACATTCTCGTTTACCTCCACAG-3′ (forward) and 5′-AAAATCACAACTGCCTTAAAGAC-3′ (reverse) for *CDKC;1*; 5′-ACCCAGCCACAACTTCTATG-3′ (forward) and 5′-CTAGTATCACATTAAATGTAAGAGTAAG-3′ (reverse) for *CDKC;2*; and 5′-TGTCGTTGTAGCGTCTTATG-3′ (forward) and 5′-TCCTTCTGTCCACTTCTATC-3′ (reverse) for *cyclin T*. The amount of target cDNA used for PCR was standardized by quantification of *actin 2* transcripts present in all the samples.

In situ hybridization

Plant material was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 (12 h at 4 °C). Fixed tissues were dehydrated through a standard ethanol series and embedded in paraffin. Tissue serial sections of 10 µm were attached to coated microscope slides. ^{35}S-UTP-labeled sense (control) and antisense RNA probes were generated by in vitro transcription with T7 and Sp6 RNA polymerases, according to the manufacturer's protocol (Roche Diagnostics). *CDKC;2* DNA-coding sequence was used as template for CDKC riboprobe synthesis. Because the sequence dissimilarity between *CDKC;1* and *CDKC;2* is extremely subtle and spread throughout the gene, this riboprobe is highly unlikely to bind specifically to *CDKC;2* RNA in the tissue. On the contrary, this probe must be able to recognize both the *CDKC;1* and *CDKC;2* RNA targets. Full-length transcripts were reduced to 300-bp fragments through alkaline hydrolysis. Plant material was hybridized with the appropriate antisense and control probes as previously described [18]. Autoradiographs were taken with a Diaplan microscope (Leitz, Heerbrugg, Switzerland) using dark-field illumination.

Results

Isolation of *CDKC;1* and *CDKC;2* genes and sequence analysis

Arath;*CDKC*;*2* kinase was first identified as an EST based on its sequence similarity to other known CDKs. Using the EST sequence information, a full-length cDNA was cloned. The cDNA (1738 bp) encodes a relatively long CDK protein of 505 amino acids (56.7 kDa predicted molecular mass). Based on sequence homology, a second *Arabidopsis* gene coding for a putative CDKC protein (CDKC;1) was identified. Both genes are located on chromosome V and are predicted to be the result of gene duplication [19]. *CDKC;1* and *CDKC;2* gene sequences are 86% identical, whereas the encoded amino acid sequences share 92% similarity. These CDKC proteins are homologous to CDKs identified in alfalfa, tomato, and pea (approximately 80% identity), all of which bear a PITAIRE signature motif in the cyclin-binding domain [20–22]. In addition to their protein kinase domain, CDKC;1 and CDKC;2 present a nuclear localization signal at the C-terminal end (position 350– 367) (fig. 1). Comparison of the *Arabidopsis* CDKC sequences with all known non-plant CDK proteins showed that these proteins are closely related to CDK9 (50% identity) (fig. 1).

Two-hybrid protein interaction assays

To understand the possible function of the Arath;CDKC;2 protein, a yeast two-hybrid screen was undertaken to search for its protein partners. One of the interacting clones identified encodes a protein (designated *Arabidopsis* cyclin T, or AtCycT) with sequence similarity to animal cyclins from the T and K group (fig. 2A). The evolutionary relationship between animal cyclins from the T and K group and their potential plant counterparts is represented by a phylogenetic tree in figure 2B. Sequence comparison of the *Arabidopsis* cyclin T-like proteins with related proteins from different kingdoms illustrates that structurally these proteins are not directly linked to the cyclin T or cyclin K groups. The phylogenetic analysis shows that these two cyclin groups do not include plant members. On the contrary, *Arabidopsis* cyclins form a separate group sharing common structural characteristics with both the T and K cyclins. Presumably, the *Arabidopsis* T-like proteins take up the role of both the T and K animal cyclins. Alternatively, the T-like *cyclin*

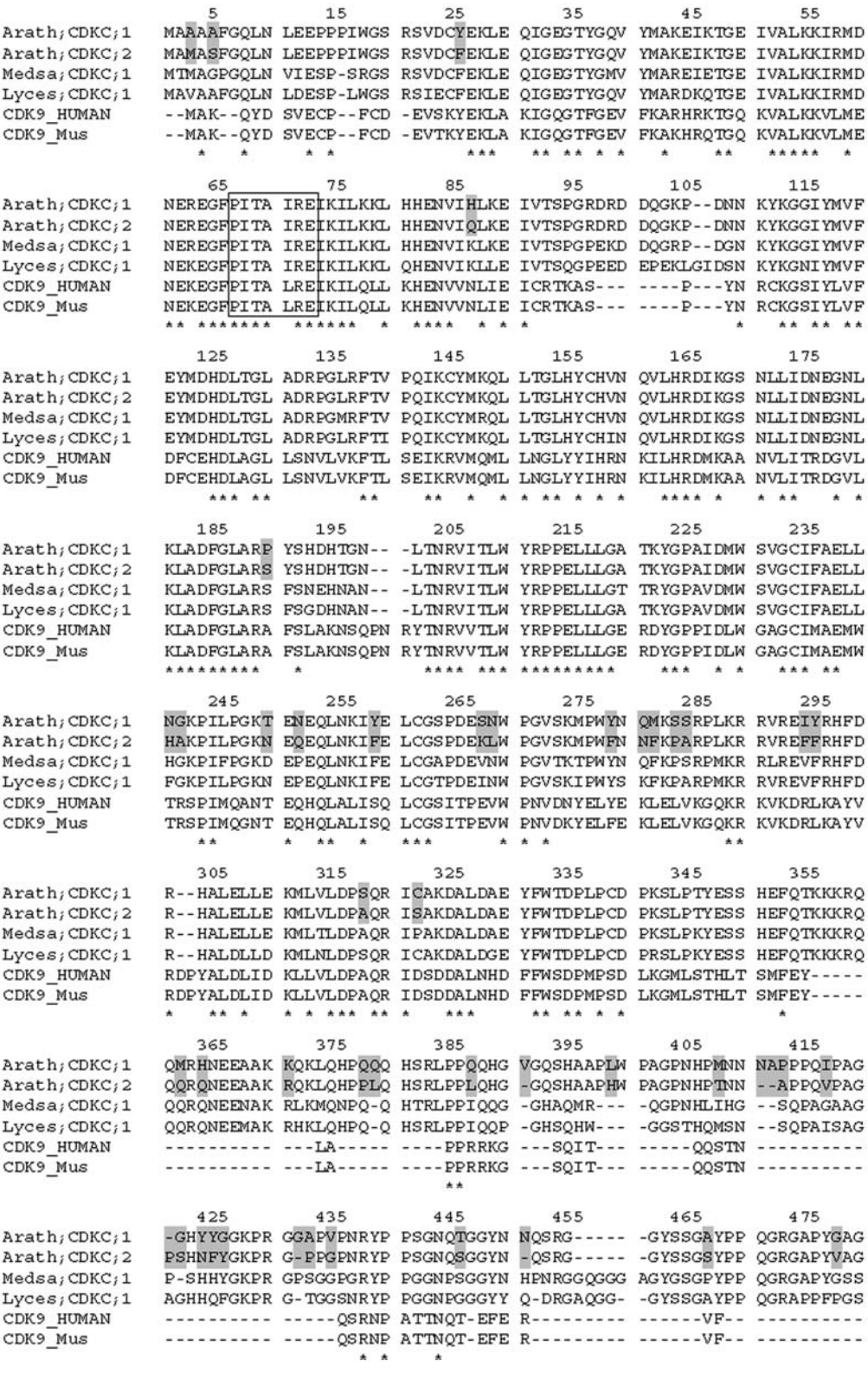

Figure 1. Amino acid sequence alignment of CDK-like proteins related to the animal CDK9. Arath;CDKC;1, CDKC;1 protein from *Arabidopsis thaliana*; Arath;CDKC;2, CDKC;2 protein from *A. thaliana*; Medsa;CDKC;1, CDK protein from alfalfa (*Medicago sativa*); Lyces;CDKC;1, CDK protein from tomato (*Lycopersicon esculentum*); CDK9_HUMAN, CDK9 protein from *Homo sapiens*; CDK9_Mus, CDK9 protein from mouse (*Mus musculus*). The amino acid residues common to the six aligned proteins are indicated with asterisks, and the characteristic PITA(L/I)RE motif is boxed. The shadowed regions in the CDKC;1 and CDKC;2 proteins correspond to the amino acid residues they do not share.

A

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                    5         15         25         35         45         55
AtCycT          MGEEHPRKRS RQHFEAEARN VSLFESP--- --------QC ETSKWYFSRE EIERFSPSRK
AtCycT-like1    MAGVLAGECS YSESGVSSHS RNSHEK---- --------QE EVSRWYFGRK EIEENSPSRL
AtCycT-like2    MAGVLAGDCS FGESGVSSYS RNSNEK---- --------QD EVARWYFGRK EIEENSPSRL
HsCycT1         ---------- ---------- -MEGERK--- ---------N NNKRWYFTRE QLE-NSPSRR
HsCycT2a        ---------- ---------- -MASGR---- ---------G ASSRWFFTRE QLE-NTPSRR
HsCycT2b        ---------- ---------- -MASGR---- ---------G ASSRWFFTRE QLE-NTPSRR
MusCycT1        ---------- ---------- -MEGERK--- ---------N NNKRWYFTRE QLE-NSPSRR
DmCycT          MSLLATPMPQ AATASSSSSA SAAASASGIP ITANNNLPFE KDKIWYFSND QLA-NSPSRR
EcCycT1         ---------- ---------- -MEGERK--- ---------N NNKRWYFTRE QLE-NSPSRR
HsCycK          ---------- MKENKENSSP SVTSAN---- -------LDH TKPCWYWDKK DLA-HTPSQL
MusCycK         ---------- ---------- ---------- -------MDH TKPCWYWDKK DLA-HTPSQL
DmCycK          ---------- ---------- ---------- ---------- -MPCWYYDKK ELR-ETPSIL
                                                             *          **

                    65         75         85         95        105        115
AtCycT          DGIDLVKESF LRSSYCTFLQ RLGMKLHVSQ VT-------- ISCAMVMCHR FYMRQSHAKN
AtCycT-like1    DGIDLKKETY LRKSYCTFLQ DLGMRLKVFP ISPQVT---- IATAIIFCHR FFFRQSHAKN
AtCycT-like2    DSIDLKKETY LRKSYCTFLQ DLGMRLKVTV LR-------- -----LLLSP FSMLSSLLT-
HsCycT1         FGVDPPDKELS YRQQAANLLQ DMGQRLNVSQ LT-------- INTAIVYMHR FYMIQSFTQF
HsCycT2a        CGVEADKELS CRQQAANLIQ EMGQRLNVSQ LT-------- INTAIVYMHR FYMHHSFTKF
HsCycT2b        CGVEADKELS CRQQAANLIQ EMGQRLNVSQ LT-------- INTAIVYMHR FYMHHSFTKF
MusCycT1        FGVDSDKELS YRQQAANLLQ DMGQRLNVSQ LT-------- INTAIVYMHR FYMIQSFTQF
DmCycT          CGIKGDDELQ YRQMTAYLIQ EMGQRLQVSQ LC-------- INTAIVYMHR FYAFHSFTHF
EcCycT1         FGLDPPDKELS YRQQAANLLQ DMGQRLNVSQ LT-------- INTAIVYMHR FYMIQSFTQF
HsCycK          EGLDPATEAR YRREGARFIF DVGTRLGLHY DT-------- LATGIIYFHR FYMFHSFKQF
MusCycK         EGLDPATEAR YRREGARFIF DVGTRLGLHY DT-------- LATGIIYFHR FYMFHSFKQF
DmCycK          DGISFETERR YRKEGARFIM ECGTKMGLGH NT-------- MATGVVYFHR FYMFHSFRSF
                       *    *          *                              *    *

                   125        135        145        155        165        175
AtCycT          DWQTIATSSL FLACKAEDEP CQLSSVVVAS YEIIYEWDPS ASIRIHQTEC YHEFKEIILS
AtCycT-like1    DRRTIATVCM FLAGKVEETP RPLKDVIFVS YEIINKKDPG ASQKIKQKEV YEQQKELILN
AtCycT-like2    --QTIATVCM FLAGKVEETP RPLKDVIVVS YEIIHKKDPT TAQKIKQKEV YEQQKELILN
HsCycT1         PGNSVAPAAL FLAAKVEEQP KKLEHVIKVA HTCLHPQES- --LPDTRSEA YLQQVQDLVI
HsCycT2a        NKNIISSTAL FLAAKVEEQA RKLEHVIKVA HACLHPLEP- --LLDTKCDA YLQQTQELVI
HsCycT2b        NKNIISSTAL FLAAKVEEQA RKLEHVIKVA HACLHPLEP- --LLDTKCDA YLQQTQELVI
MusCycT1        HRYSMAPAAL FLAAKVEEQP KKLEHVIKVA HTCLHPQES- --LPDTRSEA YLQQVQDLVI
DmCycT          HRNSMASASL FLAAKVEEQP RKLEHVIRAA NKCLPP---- -----TTEQN YAELAQELVF
EcCycT1         HRNSVAPAAL FLAAKVEEQP KKLEHVIKVA HACLHPQES- --LPDTRSEA YLQQVQDLVI
HsCycK          PRYVTGACCL FLAGKVEETP KKCKDIIKTA RSLLND---- ----VQFGQF GDDPKEEVMV
MusCycK         PRYVTGACCL FLAGKVEETP KKCKDIIKTA RSLLND---- ----VQFGQF GDDPKEEVMV
DmCycK          PRYVTACCCL FFAGKVEETP KKCRDIIKTA RGILTD---- ----NYFYSF GDDPKEEVMT
                         * * * *

                   185        195        205        215        225        235
AtCycT          GESLLLSTSA FHLDIELPYK PLAAALNRLN ---AWPDLAT AAWN----FV HDWIRTTLCL
AtCycT-like1    GEKIVLSTLG FDLNVYHPYK PLVEAIKKFK V--AQNALAQ VAWN----FV NDGLRTSLCL
AtCycT-like2    GEKIVLSTLG FDFNVYHPYK PLVEAIKKFK V--AQNALAQ VAWN----FV NDGLRTSLCL
HsCycT1         LESIILQTLG FELTIDHPHT HVVKCTQLVR ---ASKDLAQ TSYFMAT--- NSLHLTTFSL
HsCycT2a        LETIMLQTLG FEITIEHPHT DVVKCTQLVR ---ASKDLAQ TSYFMAT--- NSLHLTTFCL
HsCycT2b        LETIMLQTLG FEITIEHPHT DVVKCTQLVR ---ASKDLAQ TSYFMAT--- NSLHLTTFCL
MusCycT1        LESIILQTLG FELTIDHPHT HVVKCTQLVR ---ASKDLAQ TSYFMAT--- NSLHLTTFSL
DmCycT          NENVLLQTLG FDVAIDHPHT HVVRTCQLVK ---ACKDLAQ TSYFLAS--- NSLHLTSMCL
EcCycT1         LESIILQTLG FELTIDHPHT HVVKCTQLVR ---ASKDLAQ TSYFMAT--- NSLHLTTFSL
HsCycK          LERILLQTIK FDLQVEHPYQ FLLKYAKQLK ---GDKNKIQ KLVQMAWTFV NDSLCTTLSL
MusCycK         LERILLQTIK FDLQVEHPYQ FLLKYAKQLK ---GDKNKIQ KLVQMAWTFV NDSLCTTLSL
DmCycK          LERILLQTIK FDLQVEHPYT FLLKYAKCFK ---GDQQKLQ KMVQMAWNFV NDSLSTVVCL
                 *   * *   *      *                                    *    *

                   245        255        265        275        285        295
AtCycT          QYKPHVIATA TVHLAATFQN AKVG-SRRD- ------WWLE FG--VTTKLL KEVIQEMCTL
AtCycT-like1    QFKPHHIAAG AIFLAAKFLK VKLP-SDGE- ----KVWWQE FD--VTPRQL EDVSNQMLEL
AtCycT-like2    QFKPHHIAAG AIFLAAKFLK VKLP-SDGE- ----KVWWQE FD--VTPRQL EDVSNQMLEL
HsCycT1         QYTPPVVACV CIHLACKWSN WEIP-VSTD- ---GKHWWEY VDATVTLELL DELTHEFLQI
HsCycT2a        QYKPTVIACV CIHLACKWSN WEIP-VSTD- ---GKHWWEY VDPTVTLELL DELTHEFLQI
HsCycT2b        QYKPTVIACV CIHLACKWSN WEIP-VSTD- ---GKHWWEY VDPTVTLELL DELTHEFLQI
MusCycT1        QYTPPVVACV CIHLACKWSN WEIP-VSTD- ---GKHWWEY VDATVTLELL DELTHEFLQI
DmCycT          QYRPTVVACF CIYLACKWSR WEIP-QSTE- ---GKHWFYY VDKTVSLDLL KQLTDEFIAI
EcCycT1         QYTPPVVACV CIHLACKWSN WEIP-VSTD- ---GKHWWEY VDATVTLELL DELTHEFLQI
HsCycK          QWEPEIIAVA VMYLAGRLCK FEIQ-EWTS- KPMYRRWWEQ FVQDVPVDVL EDICHQILDL
MusCycK         QWKPEIIAVA VMYLAGRLCK FEI------- ---------- ---------- ----------
DmCycK          QWEPEIIAVA LIHLASKLSK FTVQ-DWEGR QPQQQRWWDM FVSDVTMEIL EDICHQVLDL
                    *                **                              *
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Figure 2. Sequence comparison of the *Arabidopsis* cylin T protein with related proteins from different kingdoms. (*A*) Amino acid sequence alignment of cyclin T, cyclin K, and related cyclin T-like proteins from *Arabidopsis* (AtCycT, AtCycT-like1, and AtCycT-like2). Cyclins from the T group include *Homo sapiens* cyclin T1 (HsCycT1), cyclin T2a (HsCycT2a), and cyclin T2b (HsCycT2b); *Mus musculus* cyclin T1 (MusCycT1); *Drosophila melanogaster* cyclin T (DmCycT); and *Equus caballus* cyclin T1 (EcCycT1). Cyclins from the K group include *H. sapiens* cyclin K (HsCycK); *M. musculus* cyclin K (MusCycK); and *D. melanogaster* cyclin K (DmCycK). The amino acid residues common to all protein sequences are decked out with asterisks. The C-terminal protein regions were omitted from the alignment due to the extensive sequence dissimilarities in that region. Except for the cyclins T1 that are characterized by large histidine repeats, all the proteins present very unequal C-terminal regions. (*B*) Neighbor-joining tree of the T, K, and their structurally related C and L groups of cyclin proteins with Poisson correction for evolutionary distance calculation. The scale indicates evolutionary distance. CycT, cyclin T (or T-like) proteins; CycK, cyclin K proteins; CycC, cyclin C (or C-like) proteins; CycL, cyclin L (or L-like) proteins. At, *Arabidopsis*; Ds, *Drosophila melanogaster*; Ec, *Equus caballus*; Gg, *Gallus gallus;* Hs, *Homo sapiens*; Mus, *Mus musculus*; Os, *Oryza sativa*; Sc, *Schizosaccharomyces pombe*; and Zm, *Zea mays*.

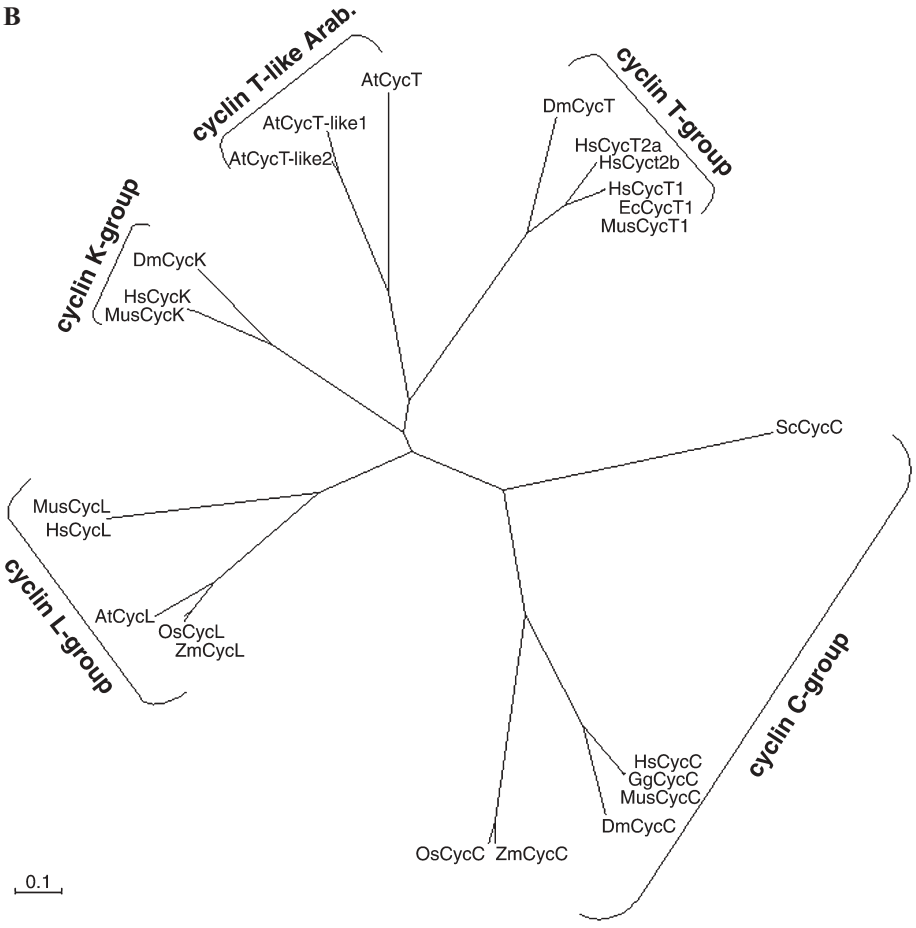

Figure 2 (continued)

genes could have undergone extensive changes during evolution, so that they can no longer be recognized as functional homologues of the animal counterparts. However, the two-hybrid interaction of the *Arabidopsis* cyclin T with a protein related to CDK9 tends to exclude this hypothesis. Therefore, T-like proteins from *Arabidopsis* might have gone through particular modifications during evolution, which allow one single protein to assume the role of both the animal cyclin T and K groups.

The two-hybrid analysis showed that Arath;CDKC;2 interacts with other proteins, such as a ribonucleoprotein (RNP), a DNA-binding protein (GT-1), and a DAG-homologous protein.

To confirm the results of the screen and to assess the specificity of the interactions, we performed additional pairwise two-hybrid assays. Vectors containing Arath;*CDKC;2*, Arath;*CDKA;1*, or Arath;*CDKB1;1* fused to the *GAL4*-binding domain were co-introduced into a yeast reporter strain together with plasmids encoding the *Arabidopsis* cyclin T protein fused to the GAL activation domain. Figure 3 illustrates that the cyclin T protein can interact with Arath;CDKC;2, but not with Arath;CDKA;1 or Arath;CDKB1;1.

In vitro transcription/translation and immunoprecipitation

To validate the two-hybrid protein interaction results, we tested the ability of CDKC;1 and CDKC;2 to heterodimerize in vitro with cyclin T and DAG-like proteins. For this purpose, HA-tagged CDKC;1 and CDKC;2, and *c-myc*-tagged cyclin T and DAG-like proteins were produced using the coupled in vitro transcription/translation system. One part of each sample was resolved by SDS-PAGE (fig. 4A, B), while the other part was subjected to immunoprecipitation with monoclonal anti-HA antibodies (fig. 4C, D). In the absence of CDKC;1 and CDKC;2 proteins, no cyclin T (fig. 4D, lanes 5–7) or DAG-like protein was precipitated by the anti-HA antibodies (data not shown). However, both cyclin T and DAG-like proteins co-precipitated reproducibly with HA-tagged CDKC;1 and CDKC;2 (fig. 4C). Identical results were obtained in a reciprocal experiment with anti-*c-myc* monoclonal antibodies (data not shown). These data revealed that both *Arabidopsis* CDKC proteins interacted in vitro with the cyclin T and DAG-like proteins, as revealed by two-hybrid interaction analysis.

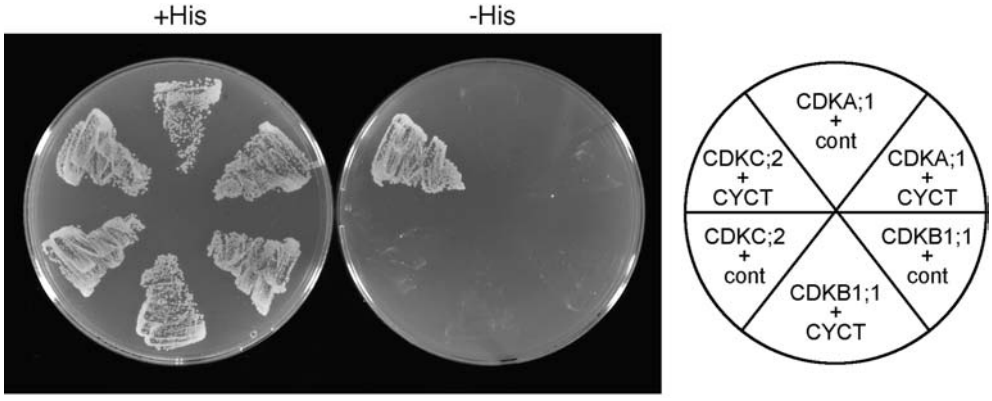

Figure 3. Yeast two-hybrid interaction of CDK proteins (CDKA;1, CDKB1;1, and CDKC;2) with the cyclin T (CYCT) from *Arabidopsis*. HF7c transformant cells of yeast were streaked on plates with (His^+) and without (His^-) histidine. Reconstitution of the GAL4 activity in the positive transformants restored the ability of yeast to grow in histidine-deficient medium. This shows that the plant cyclin T homologous protein can interact with Arath;CDKC;2 but not with Arath;CDKA;1 or Arath;CDKB1;1. cont, control two-hybrid assay performed with an empty pGBT9 vector.

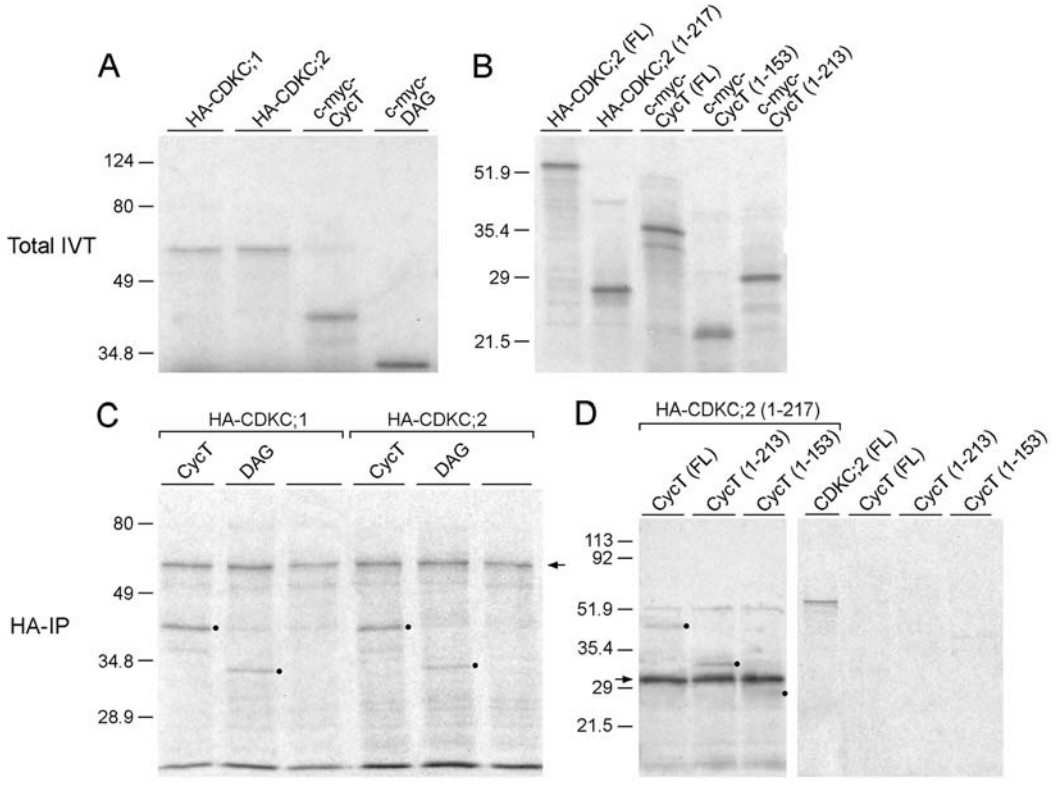

Figure 4. CDKC in vitro protein interactions. (*A*) Total in vitro translation (total IVT) extracts of the HA-tagged CDKC;1 (HA-CDKC;1) and CDKC;2 (HA-CDKC;2), as well as the *c-myc*-tagged cyclin T (*c-myc*-cycT) and DAG-like protein (*c-myc*-DAG). (*B*) The full-length versions of both CDKC;2 and cyclin T (lane 1 and 3, respectively), as well as the in vitro-translated deleted forms of HA-CDKC;2^{1-217} (lane 2), *c-myc*-cycT$^{1-153}$ (lane 4) and *c-myc*-cycT$^{1-213}$ (lane 5) are shown. Aliquots (2.0 and 2.5 µl, *A* and *B*, respectively) of each sample were directly analyzed by SDS-PAGE and autoradiographed. Another aliquot of the same samples was mixed and subjected to immunoprecipitation with anti-HA monoclonal antibodies (*C, D*). (*C*) Immunoprecipitation of cyclin T and DAG proteins by HA-CDKC;1 (lane 1 and 2) and HA-CDKC;2 (lane 4 and 5). An arrow marks the position of HA-CDKC;1 (lanes 1–3) and HA-CDKC;2 (lanes 4–6); dots mark the position of cyclin T (lanes 1 and 4) and DAG (lanes 2 and 5) proteins. (*D*) Immunoprecipitation of cyclin T full-length and mutant forms by HA-CDKC;2^{1-217} are shown on the left. The positions of cyclin T full-length (lane 1) and mutant forms (lanes 2 and 3) precipitated by HA-CDKC;2^{1-217} are marked by dots. The position of HA-CDKC;2^{1-217} is indicated by an arrow. As control HA-CDKC;2 and cyclin T were subjected to immunoprecipitation with anti-HA antibodies (right side). In the absence of CDKC;2, the cyclin T full-length and truncated forms were not immunoprecipitated by the anti-HA antibodies (lanes 5–7). Molecular mass markers are indicated to the left. In the case of mutants, the numbers refer to the amino acid sequence contained in these constructs.

To further investigate the structural requirements for heterodimerization of the CDKCs and cyclin T proteins, we tested for the in vitro interaction of deleted forms of both proteins, tagged with the HA or c-myc epitope, respectively (fig. 4D). The interactions between the deleted forms of CDKC and cyclin T were analyzed in immunoprecipitation experiments with the specific anti-HA antibodies (fig. 4D). These experiments showed that the CDKC N-terminal part (amino acids 1–217, $CDKC^{1-217}$) comprising the PITAIRE-binding domain and the T loop region is sufficient to bind the full-length cyclin T protein (fig. 4D, lane 1), even if the binding efficiency might be affected. These data indicate that the truncated CDKC region might also be required for stable interaction with the cyclin T partner.

By analogy with its mammalian counterparts, the *Arabidopsis* cyclin T sequence comprises two characteristic α-helical domains, each containing five helices. The deletion of 104 amino acids at the carboxyl-terminal end (cyclin T amino acids 1–213, $cycT^{1-213}$) did not greatly influence the binding of the cyclin to CDKC (fig. 4D, lane 2). However, the further truncation to amino acid 153 ($cycT^{1-153}$) abolished or largely reduced the binding to CDKC (fig. 4D, lane 3). The first deletion removes most of the second α-helical domain, while the second deletion also destroys the first repeat. These results indicate that the protein region between amino acids 153 and 213 is required for interaction with the CDKC partner. This assumption seems valid for both CDKC proteins because differences were not observed in their individual abilities to bind the tested cyclin T protein forms (data not shown). Thus, the first of the two helical domains of cyclin T must contain the crucial sites for dimerization with the CDKC proteins.

Gene expression analysis

The expression of *CDKC;1*, *CDKC;2*, and *cyclin T* was quantified by real-time PCR. Total RNA from seedlings, roots, rosettes, stems, and inflorescences of *A. thaliana* was reverse transcribed and cDNA samples were amplified by a LightCycler PCR using gene-specific primers. The results show that *CDKC;1*, *CDKC;2*, and *cyclin T* transcripts, although present in all tested organs, were most abundant in flower tissues. Transcript levels detected in flowers for the three genes were approximately twofold higher than in all other organs tested (table 1).

CDKC expression was confirmed by Northern analysis, using an antisense riboprobe cross-hybridizing with both *CDKC* genes. Hybridization to total RNA from various tissues revealed the existence of two similarly sized bands of approximately 1.8 kb, corresponding to the *CDKC;1* and *CDKC;2* transcripts (data not shown).

A more detailed expression profile of the *CDKC* genes was obtained by in situ hybridization on sections of *Arabidopsis* tissues. This tissue expression study was also extended to radish (*Raphanus sativus*) roots, due to the small and fragile nature of *Arabidopsis* roots. The same expression signals were visualized in the roots of both species; however, we have chosen to image radish sections since their larger structure allows the easy identification of the different cell types, including the endodermis. The transcript turned out to be mainly confined to the endodermis in roots and to the epidermis in petals (both inner and outer cell layers) and sepals (only outer epidermis) (fig. 5). *CDKC* gene expression was clearly developmentally regulated in flowers. At young stages, transcripts were only visible in sepals (mainly the distal part) (fig. 5A, B). In fully mature flowers, transcripts accumulated preferentially in petals, while in sepals, the expression slowly disappeared (fig. 5C). *CDKC* transcripts were detectable in the epidermis of the anthers and the anther filaments in fully mature flowers (fig. 5C, D). *CDKC* mRNA was never detected in carpels (fig. 5C, D). However *CDKC* transcripts were observed in the outer epidermis of siliques (data not shown). In roots, *CDKC* mRNA was confined to the endodermis. Expression was never detected in developing and mature leaves or in the shoot apical meristem. These tissue expression results indicate that CDKC proteins might have a function in fully differentiated cells, rather than being involved in cell division control.

Table 1. Semi-quantitative transcript analysis by real-time RT-PCR.

Tissues	Relative amount of DNA*		
	CDKC;1	*CDKC;2*	*cyclin T*
Seedlings	141,300	12,160	17,430
Root	97,340	13,330	16,290
Rosettes	103,700	11,860	25,090
Stems	130,500	9,130	30,620
Flowers	240,600	23,480	50,140

Total RNA from seedlings, roots, rosettes, stems, and flowers of *A. thaliana* was reverse transcribed and cDNA samples were amplified in a LightCycler PCR with gene-specific primers.

* Amounts after amplification of equal amounts of target cDNA from all tested organs.

Independent experiments showed that the values are subject to maximum 20% error.

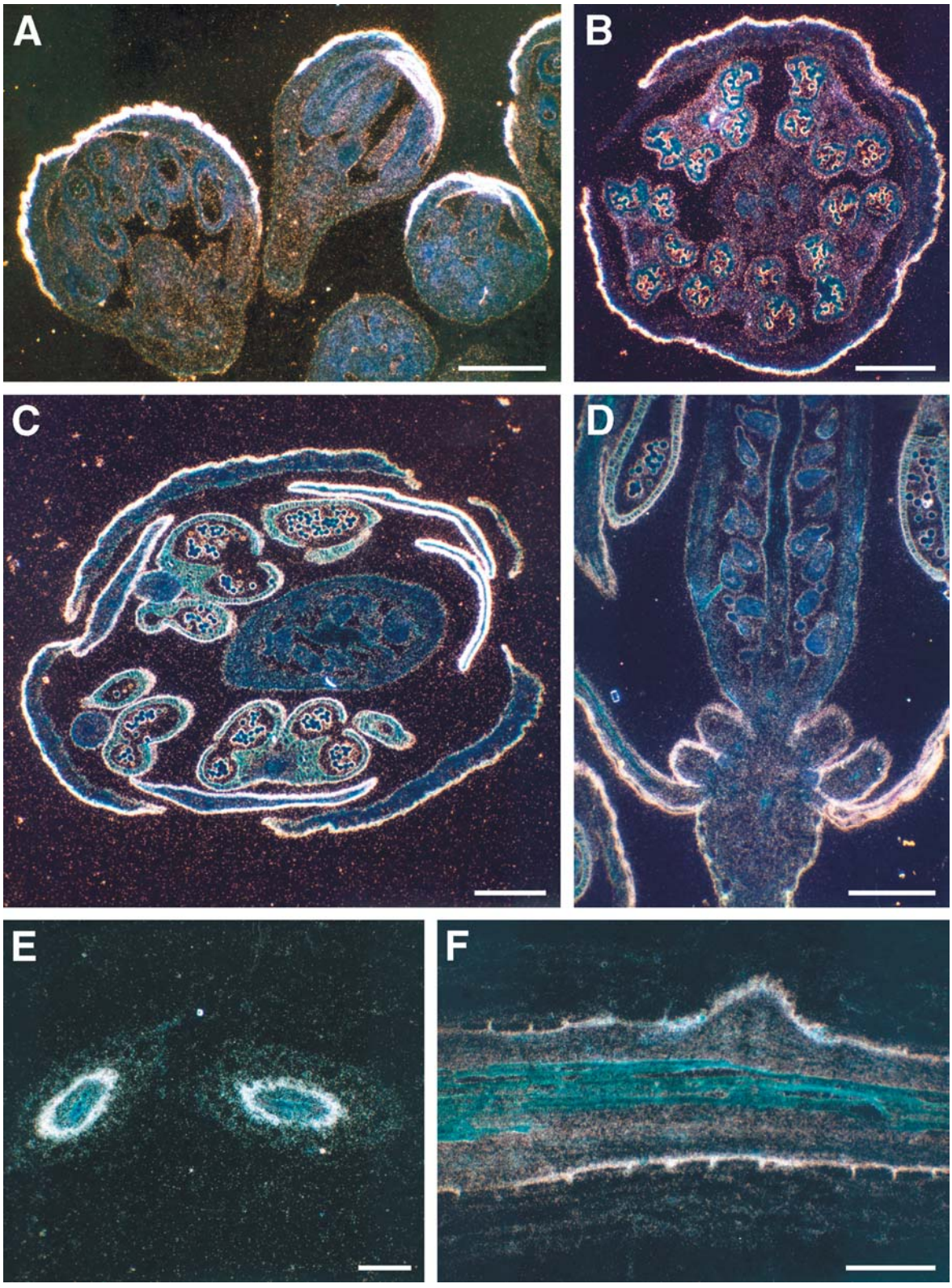

Figure 5. Arath;*CDKC;2* mRNA accumulation pattern in *Arabidopsis* flowers and radish roots, as shown by in situ hybridization. In flowers, *CDKC;2* is confined to the epidermal cells. These floral expression studies also indicated that *CDKC;2* is developmentally regulated. In young flowers, transcripts are visible only in sepals (mainly the distal part) (*A, B*), whereas in fully mature flowers, the transcripts accumulate preferentially in petals and the expression in sepals slowly disappears (*C*). In fully mature flowers, *CDKC;2* transcripts are also visible in the epidermis of the anthers and the anther filament, but never in the carpels (*C, D*). Arath;*CDKC;2* transcripts were also observed in the endodermis of radish roots (*E, F*). Scale bar, 200 µm.

To confirm a functional relationship between CDKC and cyclin T, we attempted to localize *cyclin T* transcripts in *Arabidopsis* tissues using in situ hybridization. Unfortunately, this effort proved futile, because no in situ signals were observed in any of the studied tissues (flowers and young seedlings). The failure to localize *cyclin T* mRNAs in situ might be the result of very low expression levels. Alternatively, this *cyclin* gene may be expressed in tissues other than those analyzed.

Discussion

The activity of CDKs has been widely studied in mammals and yeast. In these organisms, CDKs play a well-established role not only in the regulation of the cell cycle but also in the control of gene transcription and other regulatory mechanisms [5]. Intensive efforts over the past few years have identified many CDK homologues from plant species. However, some of these CDKs show only a limited sequence similarity to kinases of the well-characterized A and B types. Their relevance for cell cycle control is still unknown [2]. Plant kinases containing a PITAIRE motif have been identified previously, but their function has not been assigned [20, 21]. The PITAIRE motif present in Medsa;CDKC;1 of alfalfa, as well as its constitutive gene expression pattern [21] point toward Medsa;CDKC;1 as a homologue of the *Arabidopsis* CDKC protein. In tomato (*Lycopersicon esculentum*), a CDK protein (Lyces;CDKC;1) has also been identified that comprises a PITAIRE motif in its signature region. Expression studies have shown that its RNA transcripts accumulate in actively dividing tissues as well as in non-dividing cells [22].

Our results led us to hypothesize that the *A. thaliana* CDKC;2 may represent a functional homologue of the animal CDK9. CDK9 is implicated in transcription regulation as a core component of the P-TEFb complex. The P-TEFb complex is composed of a catalytic kinase subunit (CDK9) and a cyclin subunit (cyclin T1, cyclin T2, or cyclin K), as well as other as yet unidentified proteins [23]. P-TEFb has been shown to stimulate transcription elongation, through phosphorylation of the CTD domain of the largest RNAP II subunit [8, 11, 24].

In contrast to the CDKs implicated in cell cycle control, CDK9 does not phosphorylate H1. But notably, this CDK phosphorylates the C terminus of the retinoblastoma gene product [12, 25, 26]. This association of CDK9 with the retinoblastoma pathway together with its expression in terminally differentiated tissues point toward the involvement of the CDK9/cyclin T complex in determining cell differentiation rather than taking part in cell proliferation [12]. As such, the identification and characterization of CDK9 has revealed CDKs as a multifunctional group that not only governs cell division but is also implicated in pathways that promote differentiation and inhibit cell cycle progression.

When the CDK9 amino acid sequence is compared with the complete genome of *Arabidopsis*, the highest score is observed with CDKC;1 and CDKC;2. In agreement with a possible role in transcription control, CDKC proteins harbor a nuclear localization signal. Furthermore, CDKC;2 interacts with the *Arabidopsis* cyclin T that, in turn, bears the strongest similarity to the cyclin partners of CDK9. Immunoprecipitation assays confirmed the ability of the two CDKC proteins to heterodimerize with the cyclin T. Moreover, the carboxyl-terminal region of the CDKC proteins might not be required for the interaction with the cyclin T partner. On the other hand, the stable interaction of cylin T with CDKC seems to depend on the integrity of its N-terminal end, comprising the first α-helical domain. Analogous results have been reported after analyzing the binding ability of cyclin T1 mutants with CDK9, using yeast two-hybrid protein interaction assays [27]. Although kinase activity of the CDKC;2/cyclin T complex remains to be demonstrated, the specificity of interaction suggests that the two proteins are functional partners.

CDK9 may participate in many different protein complexes, each with different functional properties [23, 28]. Has Arath;CDKC;2 similar functional properties? As a first approach to address this question, we performed a two-hybrid screen to discover potential partners of CDKC proteins. Typically, most proteins identified in the screen are implicated in transcription.

Our two-hybrid results showed that Arath;CDKC;2 binds to RNPs, a DAG-like protein, a GT-1 protein, and a novel (unknown) protein with structural features shared with transcription factors. These findings strengthen the hypothesis that Arath;CDKC;2 may be a plant CDK9 homologue, belonging to a complex implicated in transcription regulation. Indeed, RNP proteins provide a functional coupling between transcription control and RNA processing [29]. RNPs are very abundant RNA-binding proteins that play an important role in the metabolism of pre-RNAs [30]. They bind to pre-mRNAs attached to RNAP II elongation complexes and influence pre-mRNA maturation, such as alternative splicing and mRNA export [31, 32]. The RNAP II CTD plays a central role in the coupling mechanism that links transcription to processing by making protein-protein contacts with both the transcription control elements and the splicing machinery [33]. This connection is established when the CTD of the RNAP II is phosphorylated, shifting the enzyme from initiation to elongation mode. The phosphorylated RNAP II domain interacts directly with factors that promote mRNA processing, recruiting them to the transcription apparatus [29]. The interaction of Arath;CDKC;2 with RNP might be essential for the regulation of diverse processing events, including mRNA splicing and transport.

The evidence that a DAG-like protein binds to Arath;CDKC;2 suggests that this kinase might regulate proteins involved in plastid development. DAG protein has been shown to be targeted to the plastids [34]. However, expression of DAG is required for the expression of nuclear genes, such as *CAB* (chlorophyll a/b-binding protein) and *RBCS* (ribulose biphosphate carboxylase), which encode proteins implicated in light-regulated gene expression [34]. Our data indicate that DAG proteins may directly interact with nuclear proteins, such as CDKC;2. We propose that a subpopulation of DAG proteins escapes chloroplast localization, being targeted to the nucleus (for instance as a complex with CDKC;2) where it probably interacts with the transcription machinery. The feasibility of such behavior has been demonstrated recently by H. Hirt [personal communication], who cloned a protein kinase that localizes in both the nucleus and the chloroplast. Another mechanism for the dual localization could be the nuclear translation of the DAG-like protein. Recent evidence indicates that up to 10–15% of the total protein in mammalian cells is synthesized in the nucleus [35, 36]. Whether DAG proteins display this type of behavior, and whether the interaction with DAG proteins implicates CDKC in the cross-talk between plastid and nuclear gene expression remains unclear.

The interaction with GT-1 supports the involvement of Arath;CDKC;2 in transcriptional control. GT-1 belongs to the class of trihelix DNA-binding proteins that attach to promoter sequences of many different plant genes. *Arabidopsis* GT-1 was found to bind, among others, to the pea RBCS and CAB2 light-responsive elements [37]. Le Gourrierec et al. [38] suggested that GT-1 activates transcription by direct interaction with transcriptional complexes. We speculate that GT-1 might interact with the transcription machinery, involving RNPs, DAG, and CDKC;2, whose activity results in the control of chloroplast protein synthesis.

An additional interacting (yet unknown) protein comprises a number of structural features characteristic for nuclear proteins involved in (chromatin-mediated) transcription regulation, including a bipartite nuclear localization signal, PEST determinants of protein instability, and plant homeodomain motifs (data not shown).

Expression analysis showed that Arath;*CDKC* transcripts are most abundant in flower tissues. mRNA in situ localization revealed that *CDKC* genes are confined to epidermal tissues. These results argue against the direct involvement of CDKCs in cell division control. On the contrary, they indicate that this protein may be involved in some specialized functions in differentiated tissues, as previously proposed for the animal CDK9.

Together, our data led us to consider that the plant CDKC/cyclin T complex is not involved in cell cycle control but rather interacts with specific components of the transcription machinery. In conformity with our expression studies and what has been shown for CDK9, we propose that CDKC is implicated in molecular mechanisms associated with cell differentiation.

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Appendix. Accession Numbers for the proteins (or putative proteins) referred to are: BAA97308 (Arath;CDKC;1), T50815 (Arath; CDKC;2), T09572 (Medsa;CDKC;1), CAC51391 (Lyces; CDKC;1), NP_570930 (CDK9_Human), P50750 (CDK9_Mus) for CDKs in figure 1; AAD46000 (AtCycT), BAB11392 (AtCycT-like1), CAB40377 (AtCycT-like2), O60563 (HsCycT1), AAC39665 (HsCycT2a), AAC39666 (HsCycT2b), AAD13656 (MusCycT1), AAF49325 (DmCycT), Q9XT26 (EcCycT1), AAH15935 (HsCycK), AAD09979 (MusCycK), XP_079707 (DmCycK) for cyclins in figure 2A, B; AF249734 (AtCycL), CAC17050 (ZmCycL), BAB39257 (OsCycL), AAD53184 (HsCycL), AAD43568 (MusCycL), NP_565622 (AtCycC), BAA13181 (OsCycC), CAC17049 (ZmCycC), CAA44720 (DmCycC), A40268 (HsCycC), AAB18947 (GgCycC), CAA19367 (SpCycC) for additional cyclins in figure 2B; AAD46000 (*Arabidopsis* cyclin T, AtCycT), G71404 [ribonucleoprotein (RNP)]; AAG09542 [DNA-binding protein (GT-1)], BAA97063 (DAG homologue protein), BAB08556 (unknown protein) for the two-hybrid CDKC;2 interacting proteins.

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